PCT

WORLD INTELLECTUAL PROPERTY ORGANIZATION
International Bureau

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 6 : C12N 9/52, 1/20

A1

(11) International Publication Number: WO 98/40473

(43) International Publication Date: 17 September 1998 (17.09.98)

(21) International Application Number: PCT/IB98/00318

(22) International Filing Date: 12 March 1998 (12.03.98)

(30) Priority Data:
9/59491 13 March 1997 (13.03.97) JP

(71) Applicant *(for all designated States except US)*: THE PROCTER & GAMBLE COMPANY [US/US]; One Procter & Gamble Plaza, Cincinnati, OH 45202 (US).

(72) Inventor; and
(75) Inventor/Applicant *(for US only)*: QUAMRUL, Hasan, A., K., M. [BD/JP]; #403 5–4–10, Uozakiminami, Higashinada–ku, Kobe 658 (JP).

(74) Agents: REED, T., David et al.; The Procter & Gamble Company, 5299 Spring Grove Avenue, Cincinnati, OH 45217 (US).

(81) Designated States: AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CU, CZ, DE, DK, EE, ES, FI, GB, GE, GH, GM, GW, HU, ID, IL, IS, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, UA, UG, US, UZ, VN, YU, ZW, ARIPO patent (GH, GM, KE, LS, MW, SD, SZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, ML, MR, NE, SN, TD, TG).

Published
With international search report.
Before the expiration of the time limit for amending the claims and to be republished in the event of the receipt of amendments.

(54) Title: KERATINOLYTIC PROTEASE EK3 AND XANTHOMONAS MALTOPHILIA EK3

(57) Abstract

The present invention provides a protease having a keratinolytic activity. The protease has the following physicochemical properties: (a) degrading caseins and keratins derived from human and animal hair, keratinolytic activity being higher than the casein degrading activity; (b) exhibiting the maximum activity under alkaline conditions of pH 10.0 and approximately 60 % of the maximum activity at the neutral pH ranges (pH 7); and (c) retaining activity substantially by 100 % at pH 10 and about by 70 % at pH 6–9, when maintained at 30 °C for 1 hour.

FOR THE PURPOSES OF INFORMATION ONLY

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

AL	Albania	**ES**	Spain	**LS**	Lesotho	**SI**	Slovenia
AM	Armenia	**FI**	Finland	**LT**	Lithuania	**SK**	Slovakia
AT	Austria	**FR**	France	**LU**	Luxembourg	**SN**	Senegal
AU	Australia	**GA**	Gabon	**LV**	Latvia	**SZ**	Swaziland
AZ	Azerbaijan	**GB**	United Kingdom	**MC**	Monaco	**TD**	Chad
BA	Bosnia and Herzegovina	**GE**	Georgia	**MD**	Republic of Moldova	**TG**	Togo
BB	Barbados	**GH**	Ghana	**MG**	Madagascar	**TJ**	Tajikistan
BE	Belgium	**GN**	Guinea	**MK**	The former Yugoslav Republic of Macedonia	**TM**	Turkmenistan
BF	Burkina Faso	**GR**	Greece			**TR**	Turkey
BG	Bulgaria	**HU**	Hungary	**ML**	Mali	**TT**	Trinidad and Tobago
BJ	Benin	**IE**	Ireland	**MN**	Mongolia	**UA**	Ukraine
BR	Brazil	**IL**	Israel	**MR**	Mauritania	**UG**	Uganda
BY	Belarus	**IS**	Iceland	**MW**	Malawi	**US**	United States of America
CA	Canada	**IT**	Italy	**MX**	Mexico	**UZ**	Uzbekistan
CF	Central African Republic	**JP**	Japan	**NE**	Niger	**VN**	Viet Nam
CG	Congo	**KE**	Kenya	**NL**	Netherlands	**YU**	Yugoslavia
CH	Switzerland	**KG**	Kyrgyzstan	**NO**	Norway	**ZW**	Zimbabwe
CI	Côte d'Ivoire	**KP**	Democratic People's Republic of Korea	**NZ**	New Zealand		
CM	Cameroon			**PL**	Poland		
CN	China	**KR**	Republic of Korea	**PT**	Portugal		
CU	Cuba	**KZ**	Kazakstan	**RO**	Romania		
CZ	Czech Republic	**LC**	Saint Lucia	**RU**	Russian Federation		
DE	Germany	**LI**	Liechtenstein	**SD**	Sudan		
DK	Denmark	**LK**	Sri Lanka	**SE**	Sweden		
EE	Estonia	**LR**	Liberia	**SG**	Singapore		

Keratinolytic Protease EK3 and Xanthomonas maltophilia EK3

BACKGROUND OF THE INVENTION

Field of the Invention

The present invention relates to a protease having keratinolytic activity under low temperatures and alkaline conditions, its use and microorganisms which produce the protease.

Background Art

Keratinases have conventionally been known to be proteases that degrade insoluble proteins such as keratins. However, all keratinases are also highly active in degrading water soluble proteins such as caseins; and, insofar as the present inventor know, there has been no report on proteases which act specifically on keratins.

On the other hand, psychrophilic bacteria have long been known, and their presence in low temperature environments has been widely recognized. They are found in soil, fish and shell fish, and dairy products, as well as in environments artificially controlled at low temperatures. Research on psychrophilic bacteria has progressed in the field of food microbiology; however, it has been based on their phylogeny and is not related to their biological features or functions.

Enzymes obtained from psychrophilic bacteria are expected to be low temperature enzymes, which are most active at cold temperatures. Such low temperature enzymes are expected to be useful as an additive in laundry detergents which would clean effectively in a cold water. Another expected use is in the food industry to improve the quality of food products which are difficult to process at high temperatures. Furthermore, the study

of enzymes derived from psychrophilic bacteria will be significant in order to elucidate the physiological functions of psychrophilic bacteria and their mechanism to adapt under low temperatures and alkaline conditions.

SUMMARY OF THE INVENTION

Recently, the present inventor isolated and purified a protease having a keratinolytic activity from the culture supernatant of Xanthomonas maltophilia EK3 and found that this protease has enzyme activity under low temperatures and alkaline conditions. The present invention is based on this finding.

Accordingly, it is an object of the present invention to provide a protease having keratinolytic activity.

It is another object of the present invention to provide a microorganism to produce the protease.

It is a further object of the present invention to provide a method for the production of the protease using the microorganism.

The protease having the keratinolytic activity according to the present invention is characterized to have the following physicochemical properties:

(a) Action and substrate specificity: Degrades caseins and keratins derived from human and animal hair. The keratinolytic activity is higher than the casein degrading activity.

(b) Optimum pH: Exhibits maximum activity under alkaline conditions of pH 10.0, and approximately 60% of the maximum activity under the near neutral conditions (pH7).

(c) pH stability: 100% of the activity is retained at pH 10.0 and about 70% is retained at pH 6-9 when maintained at 30°C for 1 hour.

(d) Optimum temperature: 50°C at pH 10.5.

(e) Temperature stability: When maintained at pH 10.5 for 1 hour, almost no activity was lost at 10°C and about 40% of activity was lost at 30°C. At temperatures higher than 50°C, activity was lost rapidly with more than 60% lost in 1 hour.

(f) Molecular weight: about 30 kDa as determined by gel chromatography.

A method for the production of the protease having the keratinolytic activity according to the present invention comprises culturing the microorganism that produces this protease and obtaining this protease from the culture medium.

Furthermore, the microorganism according to the present invention is Xanthomonas maltophilia EK3, which produces the protease having the keratinolytic activity.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 shows the results of gel filtration chromatography carried out in Example 2.

Figure 2 shows the effect of pH on the activity of the enzyme according to the present invention. ⇨:MES, ◊:MOPS, O:TAPS, Δ:CHES, ● :CAPS.

Figure 3 shows the pH stability of the enzyme according to the present invention. ⇨:Sodium citrate(pH5-7), ◊:Tris-HCl(pH7-9), O:Glycine-NaOH(pH9-11).

Figure 4 shows the effect of temperature on the activity of the enzyme according to the present invention.

Figure 5 shows the temperature stability of the enzyme according to the present invention. :10°C, ◊:20°C, O:30°C, Δ:50°C and ●:60°C.

Figure 6 shows the growth of Xanthomonas maltophilia EK3 and the activity of the produced protease, at different culture temperatures.

DETAILED DESCRIPTION OF THE INVENTION

Microorganisms

The protease having the keratinolytic activity according to the present invention can be produced using microorganisms. Any microorganism which belongs to genus Xanthomonas and produces the protease having the properties can be used for the production of the enzyme.

A preferable embodiment of a microorganism capable of producing the protease according to the present invention is Xanthomonas maltophilia EK3. This strain is a microorganism isolated by the present inventor from soil in an elephant house at a zoo, and is deposited with the accession number of FERM BP-5806 at the National Institute of Bioscience and Human-Technology of Industrial Science & Technology dated January 31, 1997.

The Xanthomonas maltophilia EK3 according to the present invention has the following microbiological properties:

(1) Morphological properties: The strain has mobility, and a spherical or spheroidal shape.

(2) Features on medium: The strain grows on an agar medium and a liquid medium, and shows white color.

(3) Optimum growth conditions: The strain grows at a temperature between 10°C and 30°C, and grows well at 20°C.

(4) Extracellularly secreted proteases: Proteases are secreted extracellularly from cells grown either at 20°C or 30°C.

(5) Aerobiosis/anaerobiosis: The stain is considered to be facultatively anaerobic as judged from biochemical tests.

(6) Gram stain: The strain is recognized to be a gram-negative bacteria according to Gram stain.

(7) Biochemical features: Primary biochemical features of Xanthomonas maltophilia EK3 are shown in Table 1.

Table 1

Test items	Results
Oxidase test	-
Oxidation-fermentation test	-
Nitrate	-
Tryptophane	-
Glucose(covered with oil)	-

Arginine Dihydrolase(covered with oil)	-
Urease(covered with oil)	-
Esculine	+
Gelatine	+
p-Nitro-Phenyl-β-D-Garactopyranoside	+
Glucose	+
Arabinose	-
Mannose	+
Mannitol	-
N-Acetyl-Glucosamine	+
Maltose	+
Gluconate	-
Caprate	-
Adipate	-
Malate	+
Citrate	+
Phenyl-Acetate	-

The isolated bacteria are identified to be a strain of Xanthomonas maltophilia (99.9%) as judged from the properties given above. The result were given by the use of Profile Index (API 20NE, NIHON Bio Merieux Biotech Co., Ltd.)

Culture of microorganisms

Bacterial cells of the strain to be used in the present invention may be cultured using either a liquid medium or solid medium. Generally, a culture with shaking or culture with stirring and aeration in a liquid medium is used.

The medium for culturing the microorganism may be any medium in which the cells can grow and produce the protease. Thus, usable carbon sources are, for example, glucose, trehalose, fructose, maltose, sucrose, starch, and malto-oligosaccharides. Usable nitrogen sources are, for example, peptone, yeast extract, malt extract, meat extract, soybean

powder, cotton seed powder, corn steep liquor, various amino acids and salts thereof, and nitrates. A synthetic medium or natural medium, which additionally contains an appropriate amount of minerals and inorganic salts, such as magnesium phosphate, calcium, sodium, potassium, iron and manganese and, if necessary, other nutrients, can be used.

Culture conditions such as pH of a medium and incubation temperature can be appropriately changed within a range in which the protease can be produced. However, when a culture with shaking or culture with stirring and aeration in a liquid medium is used, culturing at pH 10 and at 20°C is appropriate.

The protease usable in the present invention is present in the culture medium, on the cell wall and also inside the cell. Further, this protease can be used either in the form of crude enzyme secreted intracellularly or extracellularly, or extracted and purified enzyme, or directly as the cell form. Alternatively, these enzymes can be used in an immobilized form on a carrier.

Extraction and purification of enzyme

Extraction and purification of the protease of the present invention from the fluid cell culture can be carried out using known purification procedures alone or in combination.

The protease of the present invention is primarily secreted extracellularly, i.e., into the culture medium; therefore the crude enzyme fraction can be easily obtained by removing cells, for example, by filtration or centrifugation. Examples of preferable purification methods include salting out method using ammonium sulfate or the like, precipitation method using organic solvents (e.g., methanol, ethanol and acetone), absorption method using raw starch, ultra filtration, gel filtration chromatography, ion exchange chromatography and various kinds of chromatography. Embodiments of preferable purification methods will be illustrated in Examples hereinafter.

Properties of enzyme

The protease according to the present invention has the following properties:

(a) Action and substrate specificity: The protease degrades caseins and keratins derived from human and animal hair in particular. The keratinolytic activity is higher than the casein degrading activity. In other words, the protease of this invention is specific to keratins.

(b) Optimum pH: The protease exhibits the maximum activity under alkaline conditions of pH 9.5-10.5, approximately 60% of the activity obtained even at near neutral (pH 7) conditions. The optimum pH is 10.0.

(c) pH stability: When maintained at 30°C for 1 hour, 100% of the activity is retained at pH 10 and about 70% is retained at pH 6-9.

(d) Optimum temperature: Optimum temperature is 50°C at pH 10.5. About 40% of the maximum activity is retained at 40°C and about 30% of the maximum activity is retained at 20°C and 30°C.

(e) Temperature stability: When maintained at pH 10.5 for 1 hour, almost no activity was lost at 10°C and about 40% of activity was lost at 30°C. At temperatures higher than 50 °C, activity was lost rapidly with more than 60% lost in 1 hour.

(f) Molecular weight: The protease has a molecular weight of about 30 kDa as determined by gel chromatography.

Utilization of enzyme

The protease according to the present invention has a keratinolytic activity under low temperature and alkaline conditions. Accordingly, degradation of keratins, which are insoluble proteins, can be carried out at low temperatures. For example, the addition of the protease to a laundry detergent will provide a detergent which can degrade insoluble keratins. This detergent composition can be formulated according to a conventional method, except that the protease of the present invention is added. That is,

it can be produced in combination with customary detergent compositions such as surfactants for cleaning purpose,
bleaching agents and builders.

EXAMPLES

The present invention will be explained in detail by the following examples; however, the invention is not intended to be limited to these examples.

Quantitative determinations of proteins hereinafter were carried out by a pigment binding method using BioRad Protein Assay (BioRad) unless otherwise mentioned. Further, detection of proteins in chromatography was carried out by measuring absorption at a wave length of ultraviolet range, i.e., at 280 nm.

Protease activity was measured according to the following method:

(a) Phenol reagent method

A sample enzyme solution (0.2 ml) was added to 0.3 ml of 50 mM glycine-NaOH buffer (pH 10.5) containing a 1% (w/v) substrate (e.g., keratins) solution and the mixture was maintained at 30°C for 60 minutes. Then, the enzyme reaction was stopped by adding 1 ml of a trichloroacetic acid solution (0.11 M trichloroacetic acid, 0.22 M sodium acetate and 0.33 M acetic acid). After being allowed to stand at room temperature for 30 minutes, the reaction mixture was centrifuged (at 10,000 rpm, at room temperature for 10 minutes), 0.5 ml of a 2% sodium carbonate solution (0.001% copper sulfate) was added to 50 μl of the resultant supernatant and the mixture was allowed to stand for 30 minutes. To this, 50 μl of a phenol reagent solution was added after diluting in two times with distilled water. After being allowed to stand at room temperature for 1 hour, the mixture was subjected to the measurement of absorption at 660 nm.

(b) Decomposition activity of protein with azocasein

0.05 ml of a sample enzyme solution was added to 0.3 ml of a 500 mM glycine-sodium hydroxide buffer solution (pH 10.5) containing 1% (W/V) azocasein, and the mixture was then kept at 20°C for 30 minutes. Afterward, the reaction was terminated with 1 ml of a 6% trichloroacetic acid solution, and the solution was allowed to stand at room temperature for about 30 minutes and then centrifuged (15,000 rpm, room temperature, 10 minutes). Next, the absorbency of the resultant supernatant liquid at 340 nm was measured by the use of a spectrophotometer.

Example 1: Screening of novel microorganisms

The isolation of novel microorganisms was carried out on an agar plate culture medium. 0.1 g of a soil sampled at a elephant house in ISHIKAWA Prefectural Zoo was suspended in a physiological saline, and the supernatant was used as a stock solution. Furthermore, a 10^2 dilute solution of this stock solution was prepared therefrom. The stock solution and the 10^2 dilute solution were each sprayed on an agar plate culture medium for screening (25 g/liter of keratin, 1.0 g/liter of K_2HSO_4, 0.1 g/liter of NH_4NO_3, 0.1 g/liter of $MgSO_4$, 0.1 g/liter of NaCl, 0.1 g/liter of $CaCl_2$, 0.1 g/liter of $FeCl_2$, 0.1g/liter of EDTA and 15 g/liter of agar), and then cultured at 5, 10, and 20°C for 7 days. Among colonies which had grown on the agar medium, well grown colonies were selected, subcultured, and then inoculated into a retention culture medium.

It was confirmed on the agar medium that a protease was released from the bacteria. The isolated microorganisms were inoculated into the above-mentioned agar medium for screening, and then cultured at 20°C for 72 hours. Afterward, a 10% trichloroacetic acid solution was sprayed on the replica plate containing casein(1%) instead of keratin on which the bacteria grew. From the presence of transparent plaques around the colonies, it was confirmed that the protease was released from the bacteria.

In order to stabilize the growth activity of the bacteria, a bacterial strain was inoculated into 150 ml of the culture medium (each 25 ml of the medium was poured into six 100-ml Erlenmeyer flasks), and rotary shaking culture was then carried out at 20°C for 72 hours at 140 rpm by the use of a triple shaker NR-80 (Tietec Co., Ltd.). As a main culture, 150 ml of the preculture medium was inoculated into 3 liters of the culture medium, and rotary culture was then done at 10 °C for 96 hours at 140 rpm by the use of a laboratory fermenter LS-5 (Oriental Yeast Co., Ltd.).

Composition of culture medium

Keratin	2.5%(W/V)
K_2HPO_4	0.01%
NH_4NO_3	0.01%
$MgSO_4$	0.01%
NaCl	0.01%
$CaCl_2$	0.01%
$FeCl_2$	0.01%
EDTA	0.01%
(pH 10.5)	

The culture medium and the like were sterilized with high-pressure vapor for 15 minutes under 1.2 kgf/cm^2G (121°C) by an autoclave.

Example 2: Purification of enzyme

All the operations of protease purification were carried out at 4°C.

(a) Ion exchange chromatography

The culture medium obtained in Example 1 was centrifuged (8,000Xg, 4°C, 15 minutes) for separation of the bacteria and a crude enzyme. This crude enzyme solution was subjected to ion exchange chromatography to purify the same. As a column, there was used an INdEX 100 column (Pharmacia Biotec Co., Ltd.) filled with 2 liters of a DEAE Sephalose Fast Flow anion exchanger (Pharmacia Biotec Co., Ltd.). Into the above-mentioned column, a 20 mM tris-HCl buffer solution (pH 9.0) was introduced at a linear

velocity of 150 cm/hr in an amount five times (10 liters) or more as much as a gel volume to equilibrate the column.

The crude enzyme solution was introduced into the column at a linear velocity of 100 cm/hr. Elution was carried out at a linear velocity of 100 cm/hr by the use of 3 liters of each of 20 mM tris-HCl buffer solutions (pH 9.0) containing 0.2 M, 0.4 M and 0.6 M NaCl, respectively, and only portions in which a protein was detected by a UV meter were fractionated.

(b) Salting out with ammonium sulfate

Ammonium sulfate was added to the thus obtained fraction under ice cooling so that the fraction might be saturated as much as 80% with ammonium sulfate. After the solution was slowly stirred at 4°C overnight in a cold chamber, centrifugation (18,000 g, 4°C, 30 minutes) was carried out to precipitate an enzyme, thereby obtaining a saturated fraction. The amount of ammonium sulfate to be added was an amount necessary to achieve a saturated concentration at 25°C.

(c) Gel filtration

Gel filtration was carried out through a HiLoad 16/60 Superdex 200 prep grade column (Pharmacia Biotec Co., Ltd.). As a device, there was used HiLoad System 50 (Pharmacia Biotec Co., Ltd.). A tris-HCl buffer solution (pH 9.0) was caused to flow through the HiLoad 16/60 Superdex 200 prep grade column at a linear velocity of about 60 cm/hr to equilibrate the column, the amount of the tris-hydrochloric acid buffer solution being three times or more (400 ml) as much as the gel volume. Afterward, 5 ml of the sample enzyme solution which had been subjected to the salting out with ammonium sulfate was introduced into the column by the use of a Superloop. Then, elution was carried out at a linear velocity of 60 cm/hr by the use of the tris-HCl buffer solution (pH 9.0) as an

eluent to collect fractions every 5 ml. The molecular weight determined by gel filtration chromatography is shown in Fig. 1.

Example 3: Effect of pH on enzyme reaction

Degrading reaction of azocasein (1%) was carried out at various pH ranges and at 30°C for 1 hour using the enzyme purified in Example 2. Compositions of buffer solutions used for the reaction were 100 mM each of MES (2-morpholinoethanesulfonic acid hydrate) buffer (pH 5.5-6.5), MOPS (3-morpholinopropanesulfonic acid) buffer (pH 6.5-8.0), TAPS (N-tris(hydroxymethyl)methyl-3-aminopropanesulfonic acid) buffer (pH 8.0-9.0), CHES (N-cyclohexyl-2-aminoethanesulfonic acid) buffer (pH 9.0-10.0), CAPS (N-cyclohexyl-3-aminopropanesulfonic acid) buffer (pH 10.0-11.0), and glycine-NaCl-NaOH buffer (pH 11.0-13.0). Results are shown in Figure 2.

The optimum pH was revealed to be between 9.5 and 10.5. Further, approximately 50% of activity was found at 7.0. Accordingly, the enzyme of the present invention can act at a considerably wider pH range optimum at alkaline pH.

Example 4: pH Stability of enzyme

The enzyme purified in Example 2 was maintained in buffer solutions, i.e., 20 mM each of sodium citrate buffers (pH 5-7), tris-HCl buffer solutions (pH 7-9) and glycine-NaOH (pH 9-11), at 30°C for 1 hour, after which remaining protease activity against azocasein (1%) was measured.

Results are shown in Figure 3.

When maintained at 30°C for 1 hour, the activity of the enzyme according to the present invention was retained by almost 100% at pH 10, approximately 70% at pH 6-9 and 11. The remaining activity (%) was expressed referring the activity retained at pH 10 at 30 °C as 100%.

Example 5: Effect of temperature on enzyme reaction

Azocasein (1%) degrading reaction was carried out using the enzyme purified in Example 2 in a 50 mM glycine-NaOH buffer solution (pH 10.5) at

various temperatures for 1 hour. The reaction temperatures ranged from 10 °C to 70°C. Results are shown in Figure 4.

Optimum temperature for the reaction of the enzyme according to the present invention was 50°C at pH 10.5. The activity obtained was more than 30% at 30°C and about 40% at 40 °C, referring the activity obtained at 50°C as 100%.

Example 6: Temperature stability of enzyme

The enzyme purified in Example 2 was maintained at various temperatures between 10°C and 60°C for 1 hour. The change of the resulting activity against azocasein (1%) with the lapse of time is shown in Figure 5. In the Figure, :10°C, ◊: 20°C, O:30°C, Δ:50°C and ●:60°C.

When the enzyme of the present invention was maintained at pH 10.5 (glycine-NaOH) for 1 hour, activity loss was not observed at 10°C. On the other hand, about 40% of the activity was lost at 30°C. At 60°C, the activity was lost rapidly with time and the loss was more than 60% after being maintained for 20 min.

Example 7: Substrate specificity of protease

Casein, hemoglobin, and albumin were used as water soluble substrate proteins. Keratins (derived from human and animal hair), collagen, and elastin were used as water insoluble or slightly water soluble substrate proteins. Activity to degrade these substrate proteins was determined using the phenol reagent.

Results are shown in Table 2.

Table 2 Substrate Specificity of Protease EK3

Substrate	Relative activity
Casein*1	100
Keratin (derived from human hair)*2	190
Keratin (derived from animal hair)*3	275
Hemoglobin	87
Albumin	52

Collagen	30
Elastin	29

Note

*1: Purified according to the method described by Mr. Hammarsten.

*2: A product of Wako Pure Chemicals, code # K0001

*3: A product of Nakarai-Kagaku, code # 198-44

Reaction temperature: 30°C; reaction pH 10.5; reaction time: 60 minutes. All of the substrate concentrations were 1%.

The enzyme according to the invention specifically degrades keratins. In particular, the activity on keratin derived from animal hair was 2.75 times higher than that on casein.

What is claimed is:

1. A protease which is characterized to have the following physicochemical properties:

(a) Action and substrate specificity: degrading caseins and keratins derived from human and animal hair, keratinolytic activity being higher than the casein degrading activity;

(b) Optimum pH: exhibiting the maximum activity under alkaline conditions of pH 10.0 and approximately 60% of the maximum activity at the neural pH range (pH7);

(c) pH stability: retaining activity substantially by 100% at pH 10 and by about 70% at pH 6-9, when maintained at 30°C for 1 hour.

2. The protease according to Claim 1 where in the enzyme have further physicochemical properties:

(d) Optimum temperature: having an optimum temperature of 50°C at pH 10.5, and

(e) Temperature stability: being stable at 10°C without any loss of activity when maintained at pH 10.5 for 1 hour, being inactivated as much as about 40% at 30°C, and being inactivated rapidly at temperatures higher than 50°C with more than 60% lost in 1 hour.

3. The protease according to Claim 1 having a molecular weight of about 30 kDa determined by gel filtration chromatography.

4. A Microorganism belonging to genus Xanthomonas which produce the protease according to Claim 1, 2 or 3.

5. A microorganism according to Claim 4 which can grow well at temperatures between 10°C and 30°C.

6. The microorganisms according to Claim 4 which is Xanthomonas maltophilia EK3.

7. A microorganism deposited under the accession number FERM BP-5806.

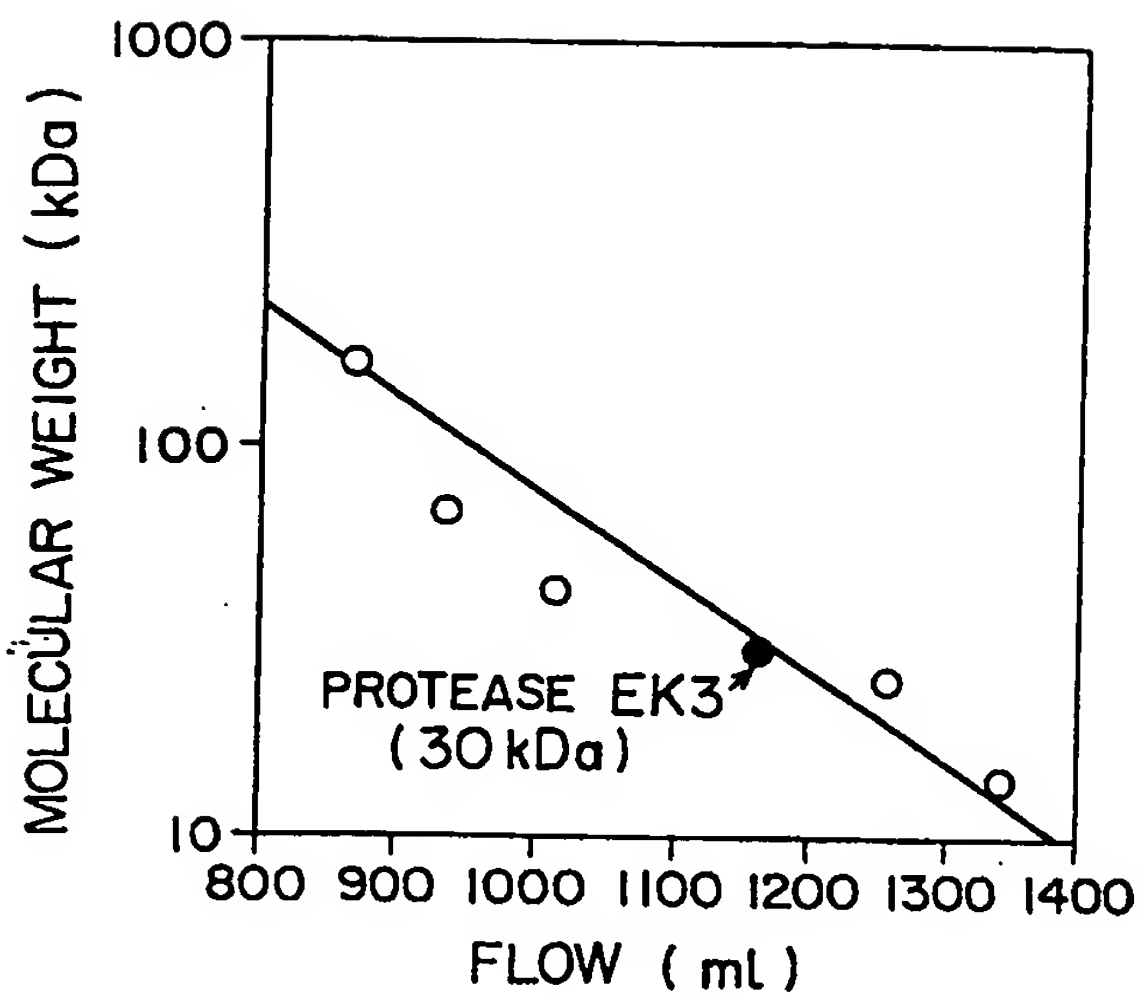
8. A method for preparing the protease according to Claim 1, 2 or 3, which comprises culturing the microorganism according to one of Claims 4-7 and obtaining the protease according to Claim 1, 2 or 3 from the culture medium.

9. A protease produced by the microorganism according to anyone of Claims 4-7 having the following physicochemical properties:

(a) Action and substrate specificity: degrading caseins and keratins derived from human and animal hair, keratinolytic activity being higher than the casein degrading activity;

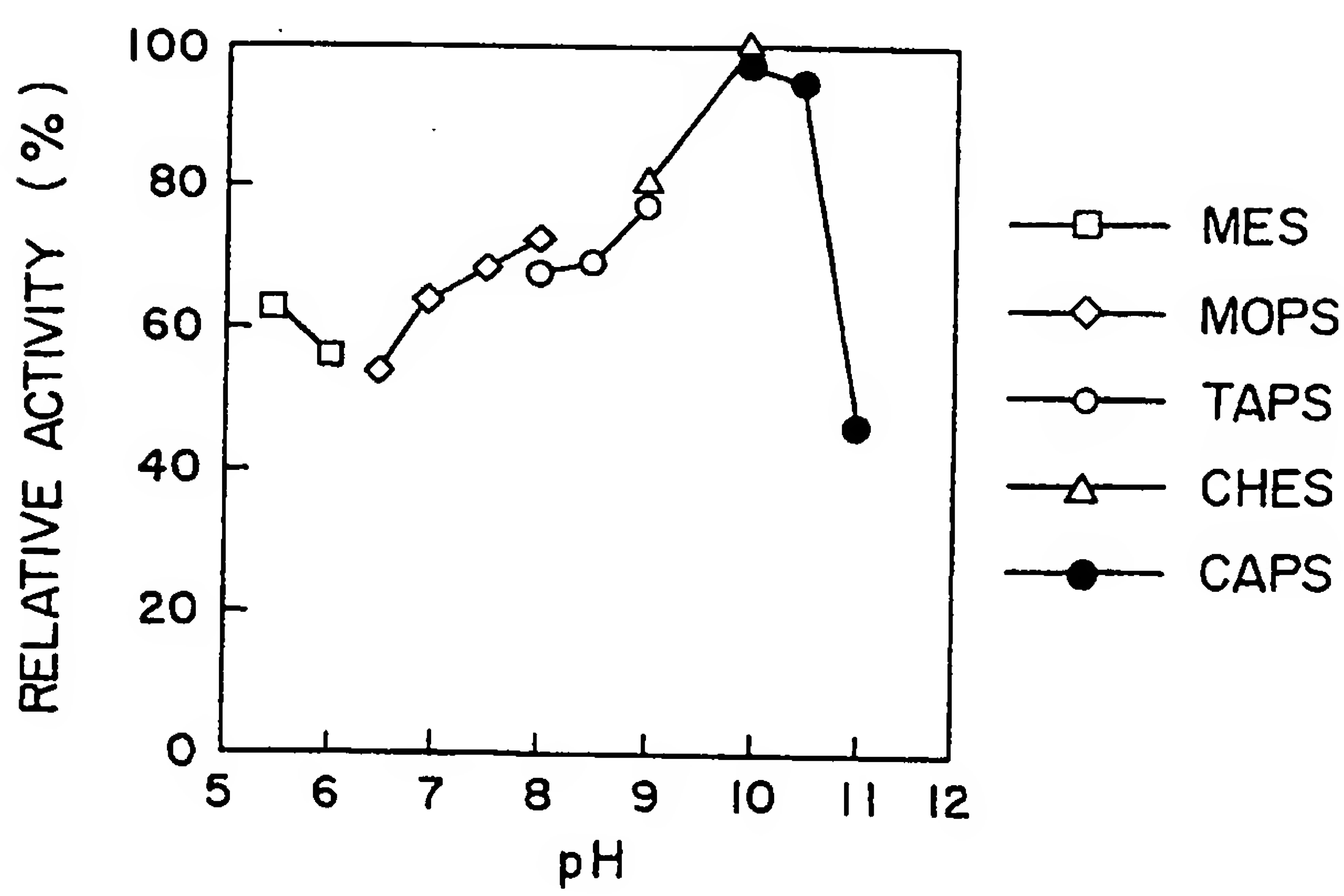
(b) Optimum pH: exhibiting the maximum activity under alkaline conditions of pH 10.0 and approximately 60% of the maximum activity at the neutral pH range (pH7);

(c) pH stability: retaining activity substantially by 100% at pH 10 and by about 70% at pH 6-9, when maintained at 30°C for 1 hour.



1000
100
10
MOLECULAR WEIGHT (kDa)
PROTEASE EK3
(30 kDa)
800 900 1000 1100 1200 1300 1400
FLOW (ml)

FIG. 1



100
80
60
40
20
0
RELATIVE ACTIVITY (%)
5 6 7 8 9 10 11 12
pH
MES
MOPS
TAPS
CHES
CAPS

FIG. 2

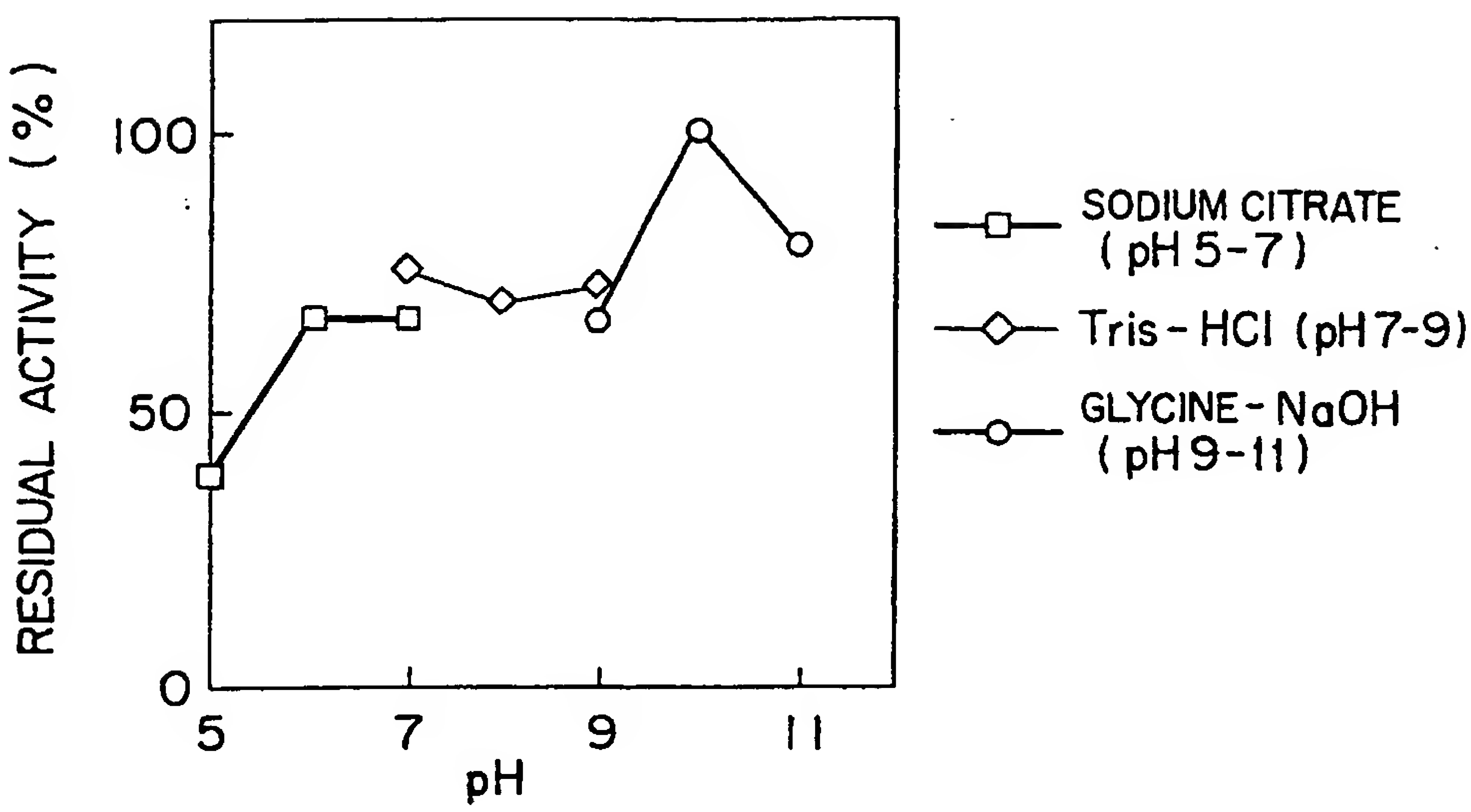

RESIDUAL ACTIVITY (%)
100
50
0
5
7
9
11
pH
SODIUM CITRATE (pH 5-7)
Tris-HCl (pH 7-9)
GLYCINE-NaOH (pH 9-11)

FIG. 3

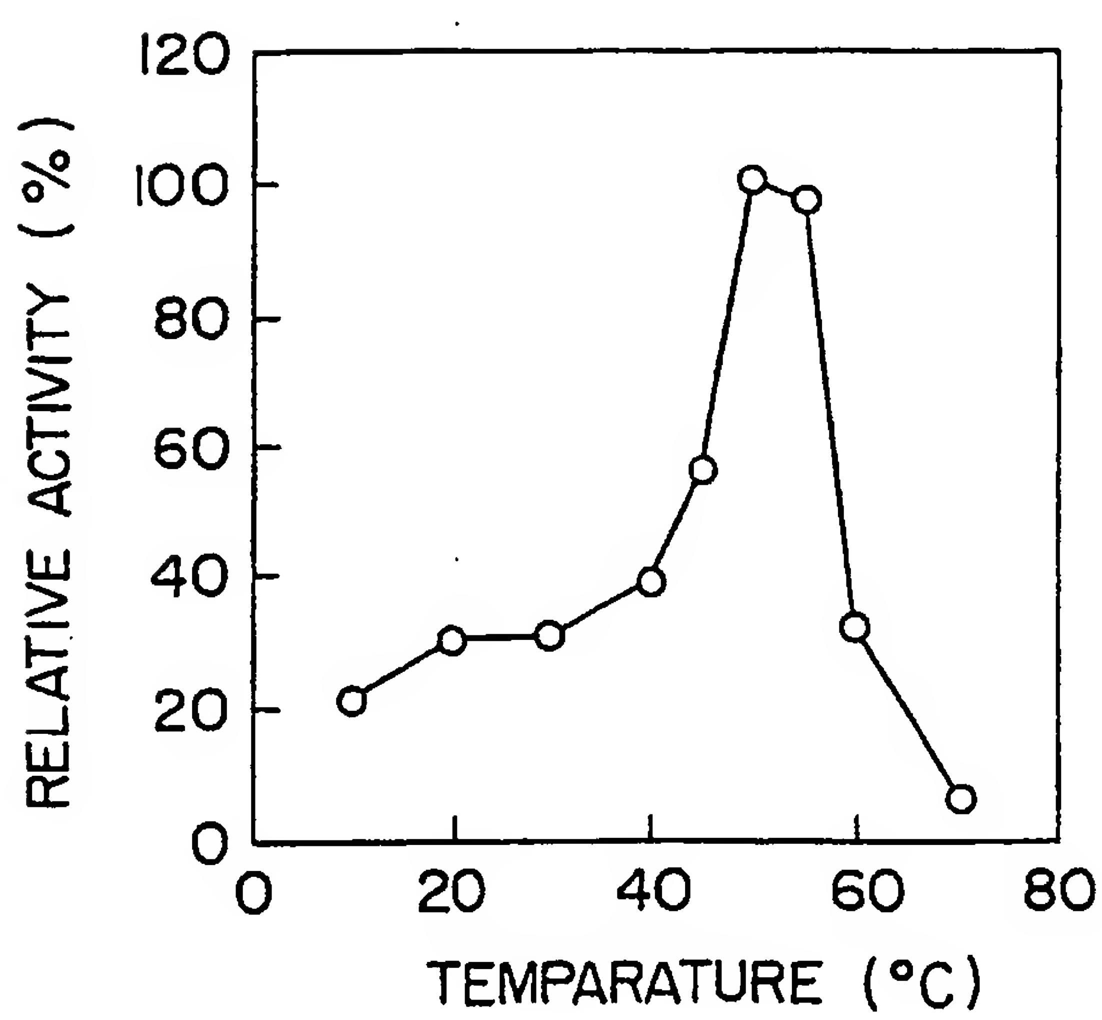
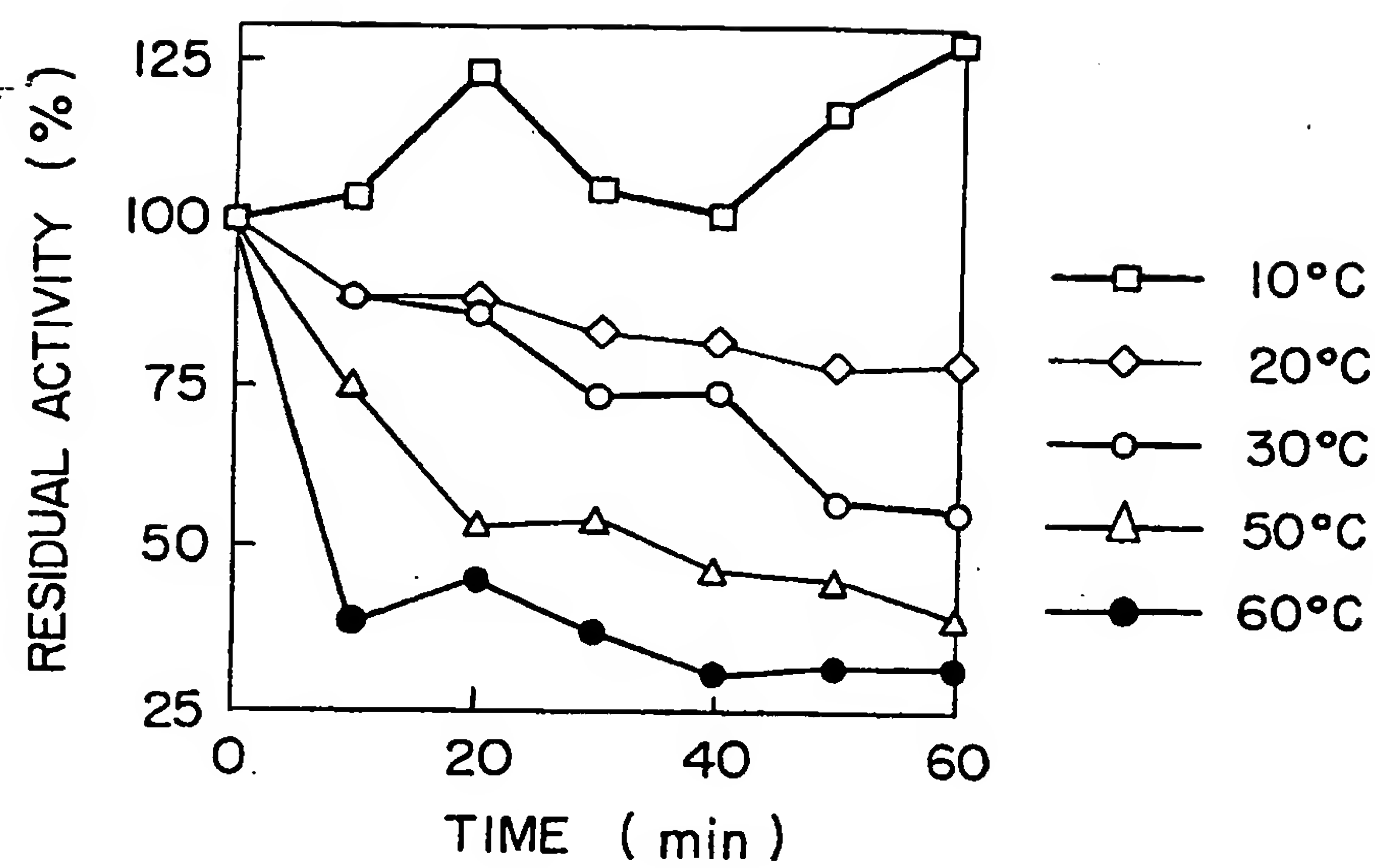

RELATIVE ACTIVITY (%)
120
100
80
60
40
20
0
0
20
40
60
80
TEMPARATURE (°C)

FIG. 4



125
100
75
50
25
RESIDUAL ACTIVITY (%)
0
20
40
60
TIME (min)
10°C
20°C
30°C
50°C
60°C

F I G. 5

4/4

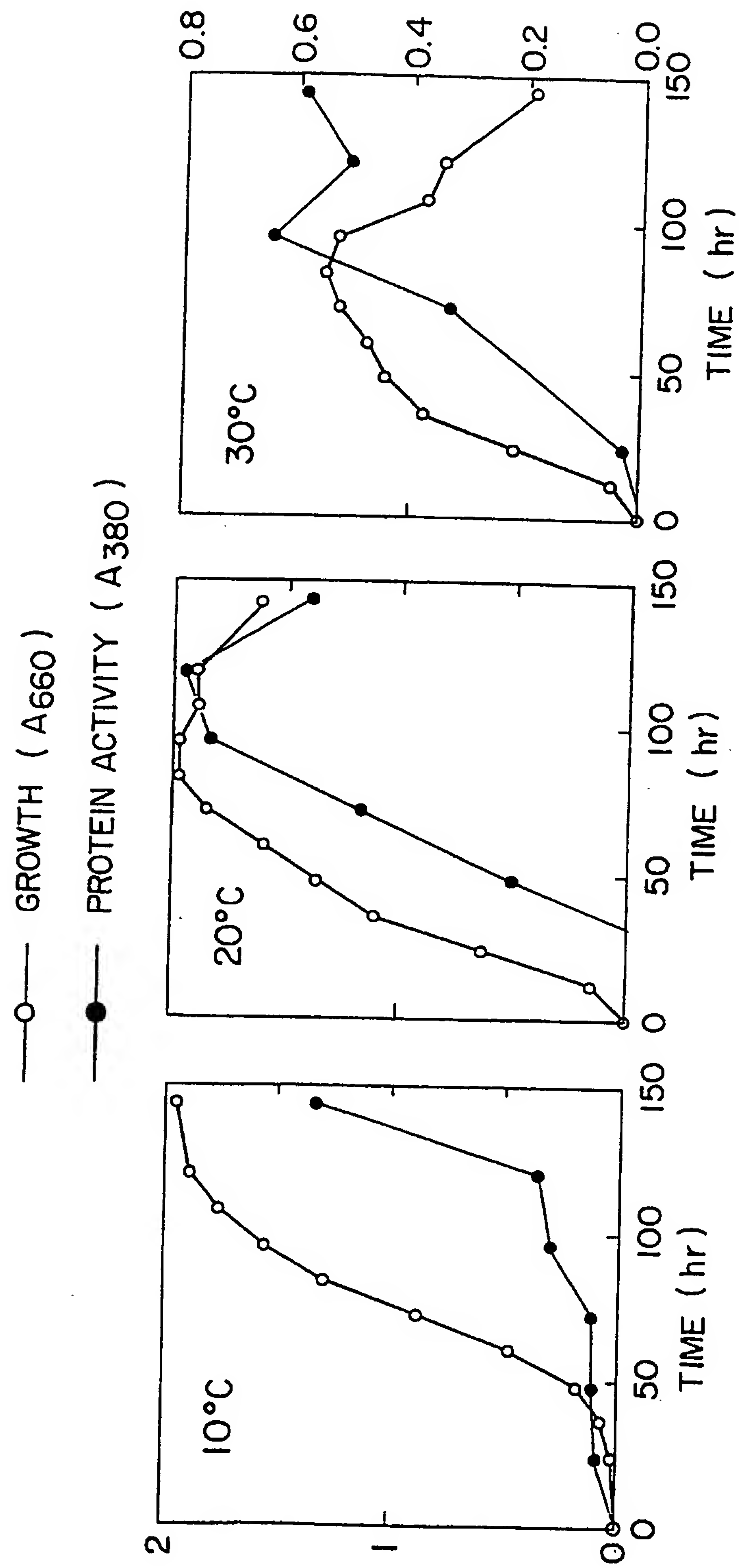

GROWTH (A660)
PROTEIN ACTIVITY (A380)
10°C
20°C
30°C
TIME (hr)
0
50
100
150
0
1
2
0.0
0.2
0.4
0.6
0.8

FIG. 6

INTERNATIONAL SEARCH REPORT

International application No.
PCT/IB98/00318

A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) :C12N 9/52, 1/20
US CL :435/252.1, 220, 219
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 435/252.1, 219, 220

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Enzyme Nomenclature, Bergey's Manual, The Prokaryotes.

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

Please See Extra Sheet.

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	MARGESIN et al. Characterization of a metalloprotease from psychrophilic Xanthomonas maltophilia. FEMS Microbiology Letters. 1991, Vol. 79, pages 257-261. See entire document	1-7
Y	SHAH, N.P. Psychrotrophs in milk: a review. Milchwissenschaft: Milk Science International. 1994, Vol. 49 No. 8., pages 432-437. See entire document.	1-7
Y	BURRELL et al. 'Characterisation of Isolates of Pseudomonas aeruginosa from sheep'. In: Australian Veterinary Journal. September 1984, Vol. 61 No. 9, pages 277-279. See entire document.	1-7
Y	NAKAJIMA et al., Purification and Properties of an Extracellular Proteinase of Psychrophilic Escherichia Freundii. European Journal of Biochemistry. 1974, Vol. 44, pages 87-96. See entire document.	1-3

[X] Further documents are listed in the continuation of Box C. [ ] See patent family annex.

* Special categories of cited documents:
"A" document defining the general state of the art which is not considered to be of particular relevance
"E" earlier document published on or after the international filing date
"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
"O" document referring to an oral disclosure, use, exhibition or other means
"P" document published prior to the international filing date but later than the priority date claimed
"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
28 MAY 1998	16 JUL 1998

Name and mailing address of the ISA/US	Authorized officer
Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231	IRENE MARX
Facsimile No. (703) 305-3230	Telephone No. (703) 308-0196

Form PCT/ISA/210 (second sheet)(July 1992)★

INTERNATIONAL SEARCH REPORT

International application No. PCT/IB98/00318

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	ALICHANIDIS et al. Some properties of the extracellular protease produced by the psychrotrophic bacterium Pseudomonas Fluorescens strain AR-11, Biochimica et Biphysica Acta. 1977, Vol. 485, pages 424-433. See entire document.	1-7
Y	PATEL et al. Heat-Stable proteases from psychrotrophic pseudomonads: secondary structure and heat stability. Food Microbiology. 1988, Vol. 5, pages 201-211. See entire document.	1-7
X	JP 5-211868 A 24 August 1993. See entire document.	1-7

Form PCT/ISA/210 (continuation of second sheet)(July 1992)★

INTERNATIONAL SEARCH REPORT

International application No.
PCT/IB98/00318

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☐ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:

2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:

3. ☒ Claims Nos.: 8-9
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.

2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.

3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:

4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

☐ The additional search fees were accompanied by the applicant's protest.

☐ No protest accompanied the payment of additional search fees.

Form PCT/ISA/210 (continuation of first sheet(1))(July 1992)★

B. FIELDS SEARCHED

Electronic data bases consulted (Name of data base and where practicable terms used):

APS, CASS ONLINE DIALOG, BIOSIS, AGRICOLA MEDLINE

search terms: protease, proteinase, proteolyt?, xanthomonas maltophila, pseudomonas, psychrophil?, cold, low temperature, casein, keratin?